Macrophage-derived reactive oxygen species suppress miR-328 targeting CD44 in cancer cells and promote redox adaptation

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CD44 is frequently overexpressed in a wide variety of epithelial malignancies including gastrointestinal cancer and causes resistance to currently available treatments. MicroRNAs (miRNAs) are non-coding RNAs that regulate molecular pathways in cancer by targeting various genes. The aim of this study was to investigate the regulation of CD44 expression by miRNAs and to develop new molecular targets in gastrointestinal cancer. We performed miRNA screening in six human gastrointestinal cancer cell lines and identified three candidate miRNAs that could regulate CD44 expression in gastrointestinal cancer. Among these, we focused on miR-328 and examined its functional relevance using growth assays and cytotoxicity assays. CD44 expression was reduced in gastrointestinal cancer cell lines forced to express miR-328, leading to inhibition of cancer cell growth in vitro and in vivo, and impaired resistance to chemotherapeutic drugs and reactive oxygen species (ROS). In contrast, induction of CD44 expression by miR-328 inhibitor led to promotion of cancer cell growth. Furthermore, we revealed that ROS produced by macrophages triggered CD44 expression through suppression of miR-328 in gastric cancer cells. Finally, tumor-infiltrating macrophages (CD68 and CD163) were closely related to both miR-328 downregulation and CD44 upregulation in 63 patients with surgically resected gastric cancer. These findings suggest that macrophages in the tumor microenvironment may be key factors in the mechanism of CD44 upregulation in gastrointestinal cancer tissues. MicroRNAs (miRNAs) play an important role in the regulation of gene expression, and their dysregulation has been implicated in various pathological processes.

Introduction

Increasing evidence has recently shown that cancer stem cells (CSCs) possess an enhanced tumor-initiating capacity, and are implicated in tumor progression and metastasis. CD44 has been identified as one of the cell surface markers associated with CSCs in several tumor types (1–3) and has been implicated in a variety of physiological processes in addition to cancer cell invasion and metastasis. CD44 is synthesized in multiple isoforms as a result of alternative messenger RNA (mRNA) splicing (4,5). We previously demonstrated abundant expression of the CD44 variant isoform (CD44v) and its association with the progression of mouse gastric tumors and human gastrointestinal malignancies (6,7). Furthermore, we reported that CD44v contributed to reactive oxygen species (ROS) defense by promoting the synthesis of the primary intracellular antioxidant glutathione. CD44v interacts with and stabilizes xCT, a subunit of the glutamate–cystine transporter, thereby promoting the uptake of cysteine for glutathione synthesis and resulting in the proliferation of gastric tumor cells (8).

MicroRNAs (miRNAs) are non-coding RNAs (20–22 nucleotides long), which repress mRNA translation by base pairing to partially complementary sequences in the 3′-untranslated region (UTR) of their target miRNAs. These non-coding RNAs have the potential to post-transcriptionally regulate ~30% of all human genes (9). Emerging evidence suggests that dysregulation of miRNAs is involved in the pathogenesis of many cancers, and that the network of miRNAs regulates CSC properties (10,11). However, the mechanisms underlying the regulation of CSC properties by miRNAs are largely unknown.

Solid tumors consist of cancer cells and various types of stromal cells, fibroblasts, endothelial cells and hematopoietic cells, mainly macrophages and lymphocytes. Monocytes recruited from the circulation differentiate into mature macrophages within the tumor microenvironment. Macrophages comprise the most abundant immune cell population in the tumor microenvironment and are responsible for the production of cytokines, chemokines and toxic intermediates such as nitric oxide and ROS. Macrophages have functional plasticity and can change their phenotype in response to various environmental factors. There are two different states of polarized macrophage activation: classically activated (M1) and alternatively activated (M2) macrophage phenotypes. Recent studies determined that M1- and M2-polarized macrophages play different functional roles in the tumor microenvironment (12,13). M1-polarized macrophages are generally considered to act as host defense effector cells, which protect the body against attack by pathogens and tumor cells. In contrast, M2-polarized macrophages are thought to contribute to tumor growth, tumor angiogenesis, extravasation of tumor cells and suppression of antitumor immunity in various types of cancers. However, more recent studies have demonstrated that macrophages show considerable diversity and plasticity, and the phenotypes of M1- and M2-polarized macrophages can be reversed under diverse pathological conditions (14,15).

It has been reported that a local inflammatory response is responsible for increased expression of CD44 and its variant CD44v (16), but no precise mechanism of CD44 upregulation has yet been determined. We suspected that the miRNA network and inflammatory cells in the tumor microenvironment may be key factors in the mechanism of CD44 upregulation in gastrointestinal cancer tissues. In this study, we investigated the role of miR-328 in the regulation of CD44 expression in gastrointestinal cancer cells, and examined the relationship between macrophages in the tumor microenvironment and the downregulation of miR-328 and upregulation of CD44. Here, we show that macrophages may promote gastric cancer cell proliferation by downregulating miR-328 and upregulating CD44, thus identifying a potential new target for gastric cancer treatments.

Materials and methods

Cell culture and treatment

The cancer cell lines AGS, KATOIII, NUGC4, HT29 and COLO201 were cultured in 5% CO2 at 37°C in RPMI 1640 supplemented with 10% fetal...
bovine serum. HCT116 cancer cells were cultured under 5% CO₂ at 37°C in Dulbecco’s modified Eagle’s medium-nutrient mixture F-12 (Sigma, St Louis, MO) supplemented with 10% fetal bovine serum. These cell lines were obtained from the Japanese Collection of Research Bioresources Cell Bank and Riken BioResource Center Cell Bank.

**RNA and miRNA isolation**

Total RNA, including miRNA, was isolated from cell lines using a mirVana miRNA Isolation Kit (Ambion, Austin, TX) and finally eluted into 100 μl of heated elution solution, according to the manufacturer’s protocol. miRNAs were extracted from formalin-fixed, paraffin-embedded normal gastric epithelium and gastric cancer tissues using a RecoverAll Total Acidic Extraction Kit for FFPE (Ambion), according to the manufacturer’s instructions. The purity and concentration of all RNA samples were evaluated by their absorbance ratio at 260/280 nm, determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE).

**miRNA PCR array**

miRNA expression was analyzed using the Human miFinder 384HC miScript miRNA PCR Array, which profiles the expression of the 372 most abundantly expressed and best characterized miRNAs in miRBase, according to the manufacturer’s instructions.

**Quantitative real-time reverse transcription–PCR**

The expression levels of miR-328 were determined by TaqMan quantitative real-time reverse transcription–PCR (qRT–PCR) using TaqMan miRNA assay kits (Ambion), according to the manufacturer’s protocol, as described previously. miR-328 expression was normalized to that of RNU6B small nuclear RNA expression. Expression levels of CD44 were quantified by SYBR Green qRT–PCR using a LightCycler 480 SYBR Green I Master (Roche Diagnostics). All qRT–PCR reactions were performed in triplicate. The relative amounts of miR-328 and CD44 were measured with the 2^(-ΔΔCT) method. All qRT–PCR reactions were performed in triplicate.

**Transfection of miRNA**

Cells were transfected with 5 nM mimic or inhibitory miR-328 (Applied Biosystems, Foster City, CA) using Lipofectamine 2000 or RNAiMax transfection reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. The specificity of the transfection was verified using a negative control mimic (Applied Biosystems). The expression levels of miR-328 were quantified 24 and 48 h after transfection, and the cells were used for subsequent experiments.

**Generation of wild-type and mutant CD44 3’-UTR**

We predicted three candidate sites in the 3’-UTR of CD44, which might be directly bound by miR-328, using the miRanda algorithm. Oligonucleotide pairs (Supplementary Table S2, available at Carcinogenesis Online) containing the miR-328-targeting sequences of human CD44 3’-UTR and the overhangs of restriction sites were annealed. Vectors containing mutated miR-328-targeting sequences of human CD44 3’-UTR were introduced into HCT116 and HCT-8 cells using Lipofectamine LTX reagents (Invitrogen). The sequences of the small interfering RNA (siRNA; chimeric RNA–DNA) duplexes (Japan Bioservice, Saitama, Japan) were as follows: CD44, 5’-AAAUUGCCGUCA GCACUUGT-3’ and 5’-GAUCGUU GUAGGGCCAUUUUTT-3’, luciferase (GL-2, control), 5’-CGUACGCGGAUACUCUGATTT-3’ and 5’-UCGAG AUAUUCCGCGUAUAGT-3’. Cells were transfected with the annealed siRNAs for 24–72 h using Lipofectamine RNAiMAX reagent (Invitrogen).

**Stable miR-328 overexpression and in vivo tumorigenicity assay**

An expression vector encoding miR-328 was constructed and introduced into the BLOCK-iTM Pol II miR RNAi Expression Vector Kit with EmGFP (Invitrogen). HCT116 cells were transfected with the resulting vectors using Lipofectamine LTEx reagents (Invitrogen). Cells stably expressing miR-328 were obtained by selection with 5 μg/ml blasticidin for 2 weeks and then isolated by FACS based on EmGFP expression. Six-week-old nude mice (Balb/c-nu/nu) were inoculated subcutaneously in the right flank with HCT116 cells (1 × 10⁶) stably expressing control or miR-328 vector in 100 μl phosphate-buffered saline containing 50% Matrigel (BD). All animal procedures and care were approved by the animal care and use committee of Kumamoto University.

**Western blot analysis**

Cultured cells collected from six-well plates were washed once in phosphate-buffered saline and lysed in radioimmunoprecipitation buffer supplemented with protease/phosphatase inhibitor cocktail (Thermo Scientific, Tokyo, Japan). Each protein sample was submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and exposed to primary antibodies. Signals were detected by incubation with secondary antibodies labeled using the Enhanced Chemiluminescence Detection System (GE Healthcare, Little Chalfont, UK).

**Immunohistochemistry and scoring**

Sample processing and immunohistochemistry (IHC) procedures were performed as described in a previous report (8). Endogenous peroxidase activity was blocked using 3% hydrogen peroxide. The sections were incubated with diluted antibodies and then with a biotin-free horseradish peroxidase-labeled polymer from the Envision Plus detection system (Dako, Glostrup, Denmark). Positive reactions were visualized using diaminobenzidine solution, followed by counterstaining with Meyer’s hematoxylin. All IHC staining was scored independently by two pathologists. The membranous CD44v9, cytoplasmic CD68 and CD163 were expressed independently in the right flank with HCT116 cells (1 × 10⁶) stably expressing control or miR-328 vector in 100 μl phosphate-buffered saline containing 50% Matrigel (BD). All animal procedures and care were approved by the animal care and use committee of Kumamoto University.

**Antibodies for IHC and immunoblotting analyses**

For IHC, human CD44 was detected using a previously generated rat monoclonal antibody specific for human CD44v9 (1:100) (8). Human CD68 was detected using a mouse monoclonal antibody specific for human CD68 (1:100 dilution; Dako). Human CD163 was detected using a mouse monoclonal antibody specific for human CD163 (1:100 dilution; Novocastra, Newcastle, UK). For immunoblot analysis, human CD44 was detected using previously generated rabbit polyclonal antibodies to CD44cyto (1:10000) (17). Human β-catenin was detected with a rabbit monoclonal antibody for human β-catenin (1:1000; Cell Signaling Technology, Tokyo, Japan). Human β-actin was detected with a rabbit polyclonal antibody for human β-actin (1:1000; Cell Signaling Technology).

**Measurement of ROS**

Cells were incubated with 10 μM 2’,7’-dichlorofluorescein diacetate for 15 min at 37°C. Washed twice with phosphate-buffered saline and subjected to fluorescence microscopy in images acquired with a Biorevo BZ-9000 fluorescence microscope (Keyence, Tokyo, Japan) and analysis software.

**Cell proliferation assay**

Cell proliferation assays were carried out in 96-well plates using the WST-8 assay with a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) at 24, 48, 72 and 96 h after transfection, according to the manufacturer’s instructions. Absorbance was measured at 450 nm.

In vitro cytotoxicity assay

HCT116 cells transfected with control or mimic miR-328 were plated in 96-well microwells (2 × 10³ cells per well), cultured overnight and then exposed to H₂O₂ or cisplatin (CDDP) in triplicate for 3 days. The number
of viable cells was then determined using a Cell Counting Kit-8 (Dojindo Laboratories). Each IC$_{50}$ value was determined as the concentration of test agent for which test luminescence/control luminescence (T/C) equaled 50%.

**THP-1 macrophage preparation and co-culture assay**

For M1-polarized THP-1 macrophages, 320nM phorbol myristate acetate (PMA) was added to THP-1 cells for 6h, followed by PMA plus 20ng/ml interferon γ and 100ng/ml lipopolysaccharide for the following 18h. For M2-polarized THP-1 macrophages, 320nM PMA was added to THP-1 cells for 6h, followed by PMA plus 20ng/ml interleukin (IL)-4/IL-13 for the following 18h. After three washes to remove cytokines, M1- or M2-polarized THP-1 macrophages (upper inserts) were co-cultured with AGS cells (in six-well plates, 2 x 10$^5$ cells per well) without direct contact. After 24 h of coculture, the upper inserts containing macrophages were discarded and the AGS cells were washed and used for subsequent experiments.

**Patients and tissue samples**

Primary gastric carcinoma tissue and matched normal gastric epithelium were obtained from 63 patients who underwent gastric resection without preoperative treatment at the Department of Gastroenterological Surgery, Kumamoto University Hospital from 2005 to 2008, after receiving adequate informed consent. The study was approved by the medical ethics committee of Kumamoto University.

**Statistical analysis**

All experiments were performed in triplicate and the data shown are representative of consistently observed results. Data are presented as the mean ± SD. Independent Student’s t-tests were used to compare continuous variables between the two groups. Data were analyzed using JMP (SAS Institute, Tokyo, Japan) and Excel 2007 (Microsoft, Redmond, WA). A P-value of <0.05 was considered statistically significant.

**Results**

**Identification of miRNAs regulating CD44 expression using cancer-related-miRNA screening in gastrointestinal cancer cells**

CD44 is a cell surface marker for CSCs and is involved in the regulation of CSC ability. We recently reported that CD44 played a functional role in ROS defense and caused tumor development in gastrointestinal cancer (8). CD44 expression is regulated by Wnt β-catenin signaling in the intestine (20). We, therefore, examined CD44 and β-catenin expression in three gastric cancer lines (AGS, NUGC4 and KATOIII) and three colorectal cancer lines (COLO201, HT29 and HCT116) by western blotting. There was a negative association between CD44 and β-catenin expression (Figure 1A). Several miRNAs are implicated in regulating the abilities of CSCs, including self-renewal, tumorigenicity and chemoresistance (11). We, therefore, tested the hypothesis that the regulation of CD44 expression in gastrointestinal cancer cells may be mediated by miRNAs using miRNA qRT–PCR array analysis. We selected 59 miRNAs that were downregulated by less than half in high-CD44-expressing cells compared with low-CD44-expressing cells (Supplementary Table S1, available at Carcinogenesis Online). We also selected all 41 miRNAs that were raised as candidates for directly targeting the human CD44 3′-UTR by using online databases (miRTarBase, TarBase, microRNA.org and TargetScanHuman). We then selected three miRNAs (miR-328, miR-373 and miR-520c) that met both these requirements (Figure 1B). Previous studies characterized miR-520c and miR-373, belonging to the miR-520/373 family, as oncogenes implicated in cancer cell migration and invasion, but with no effect on cancer cell proliferation (21).

**Fig. 1.** Identification of miR-328 directly regulating CD44 expression in gastrointestinal cancer cells. (A) Immunoblot analysis of CD44, β-catenin and β-actin (loading control) in indicated gastrointestinal cancer cell lines. (B) The left panel shows scatter plots of miRNA expression in high-CD44-expressing cells compared with low-CD44-expressing cells. The right panel shows schematic representation of three miRNAs that met the requirements for regulating CD44 expression. (C) Alignment of the three predicted miR-328 target sequences in the 3′-UTR and the mutated sequence of miR-328-targeting 3′-UTR of CD44 mRNA. The seed match sequences for miR-328 are indicated by lines. (D) Luciferase activity of HCT116 cells transfected with constructed plasmids containing wild-type or mutant miR-328 target sequences in the 3′-UTR of CD44 mRNA and co-transfected with mimic control or mimic miR-328, respectively (*P < 0.03).
miR-328, however, was recently shown to be downregulated in colorectal cancer and to regulate CSC-like side-population cells by targeting adenosine triphosphate-binding cassette subfamily G member 2 and matrix metalloproteinase 16 (22). Because CD44 is overexpressed and implicated in the proliferation of cancer cells, we therefore focused on miR-328 for further analysis.

**CD44 is a direct target of miR-328**

We investigated if miR-328 directly targeted the 3'UTR of CD44 using constructs containing the putative miR-328 target site or a mutated sequence of the 3'UTR of CD44 cloned immediately downstream of the luciferase reporter gene. We checked the predicted target sequence of miR-328 in the CD44 3'UTR using miRanda algorithm. LUC-CD44-a, -b and -c represent alignments of the predicted miR-328 target sequences in the CD44 3'UTR mRNA. Seed sequences are indicated by lines in Figure 1C. HCT116 cells transfected with a miR-328 mimic significantly suppressed luciferase activity from the reporter vectors containing the wild-type CD44 3'UTR, LUC-CD44-a, but not LUC-CD44-b and -c, compared with the control vectors (Figure 1D). We also constructed reporter vectors containing the mutated CD44 3'UTR, LUC-CD44-aM (Figure 1C). HCT116 cells transfected with a miR-328 mimic did not suppress luciferase activity from the reporter vectors containing the mutated 3'UTR of CD44, LUC-CD44-aM, compared with the wild-type 3'UTR-containing vector (Figure 1D). These results indicate that miR-328 regulated CD44 expression by directly targeting its 3'UTR, 353–374.

**CD44 expression is correlated with miR-328 expression in patients with gastric cancer**

We analyzed the levels of miR-328 expression in gastric cancer tissues and normal gastric epithelium using qRT–PCR. miR-328 expression was significantly suppressed in cancer tissues compared with normal gastric epithelium (Figure 2A). Furthermore, we compared miR-328 expression levels between high- and low-CD44v9-expressing gastric cancer tissues. High CD44v9 expression levels were identified in gastric cancer cells in 62% (39/63) of samples. Interestingly, high CD44v9 expression was significantly associated with low miR-328 expression, whereas low CD44v9 expression was significantly associated with high miR-328 expression (Figure 2B).

**miR-328 expression affects cancer cell growth and drug resistance through changes in CD44 expression**

To examine the functional relevance of miR-328 expression, we analyzed its expression levels in high-CD44-expressing cancer cell lines (KATOIII and HCT116) transfected with miR-328 mimics, using RT–PCR. miR-328 expression was significantly increased in KATOIII and HCT116 cells transfected with mimic miR-328 compared with controls (Supplementary Figure S1A and B, available at Carcinogenesis Online). Western blotting analysis revealed that CD44 protein levels, regardless of β-catenin expression, were significantly reduced in KATOIII and HCT116 cells transfected with miR-328 mimics compared with controls, as indicated by qRT–PCR analysis, suggests that miR-328 have some effect on CD44 mRNA and strongly reduces CD44 protein level. (Supplementary Figure S1C, available at Carcinogenesis Online). According to our and others’ observations, CD44 is known to be overexpressed in cancer cell lines, as well as in primary tissues, and to regulate the proliferative capacity of a variety of tumor types (8,20,23). We, therefore, hypothesized that overexpression of miR-328 in gastrointestinal cancer cells might affect cell proliferation through CD44 downregulation. We, therefore, performed proliferation assays and determined that cell growth was significantly reduced in KATOIII and HCT116 cells transfected with miR-328 mimics compared with controls (Figure 3B and D). We subsequently knocked down miR-328 in low-CD44-expressing AGS cancer cells by transfection with miR-328 inhibitor and performed cell

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**Fig. 2.** miR-328 and CD44v9 expression in human gastric mucosa and cancer tissues. (A) Expression of miR-328 in paired normal gastric mucosa and gastric cancer tissues. There was a significant difference in miR-328 expression between these two groups ($P < 0.01$). (B) Upper images show CD44v9 expression pattern in gastric cancer. Lower panel shows expression of miR-328 in high- and low-CD44v9-expressing gastric cancers. Scale bars, 100 μm. There was a significant difference in miR-328 expression between these two groups ($P < 0.01$).
miR-328 targeting CD44 as a therapeutic target

proliferation assays. AGS cells transfected with miR-328 inhibitor showed significantly increased CD44 expression and cell growth. Furthermore, AGS cells co-transfected with miR-328 inhibitor and CD44 siRNA showed significantly reduced CD44 expression and cell growth (Figure 3E and F). Given our previous observation that CD44v expression was implicated in resistance to H₂O₂ and to ROS-inducing anticancer drugs such as CDDP (8), we further investigated the viability of HCT116 cells transfected with miR-328 mimics and exposed to H₂O₂ or CDDP. HCT116 cells transfected with miR-328 mimics were significantly less resistant to H₂O₂ and CDDP than control HCT116 cells (Figure 4A). We performed further experiments with the CD44v8-10 expression construct. HCT116 cells co-transfected with mimic miR-328 and CD44v8-10 vector were significantly more resistant to H₂O₂ and CDDP than cells co-transfected with mimic miR-328 and mock vector (Figure 4B and Supplementary Figure S2A, available at Carcinogenesis Online).

To determine if miR-328 expression affected tumor cell expansion in vivo, we prepared miR-328 stably expressing HCT116 cells and examined their tumorigenicity in vivo. HCT116 cells stably expressing miR-328 showed significantly reduced CD44 expression and formed significantly smaller tumors in nude mice than cells expressing a mock vector (Figure 4C and D and Supplementary Figure S2B, available at Carcinogenesis Online). These results suggest that miR-328 plays a role in regulating CD44 expression to suppress the growth of gastrointestinal cancer cells, and reduce their antioxidative capacity and resistance to chemotherapeutic drugs.

Oxidative stress by M1- and M2-polarized THP-1 macrophages induced downregulation of miR-328 and upregulation of CD44

Tumor necrosis factor (TNF) is a major inducer of chronic inflammation and ROS and is abundant under conditions of chronic inflammation, whereas CD44 expression is induced by these stimuli at inflammatory sites (16,24). We, therefore, examined CD44 expression levels and showed that CD44 expression in AGS cells was increased by TNFα treatment or H₂O₂ treatment in a concentration-dependent manner (Figure 5A). Further, miR-328 expression was markedly suppressed in AGS cells treated with H₂O₂, but not TNFα (Figure 5B). To assess the contribution of oxidative stress to miR-328 expression further, we revealed that H₂O₂-induced miR-328 downregulation was significantly inhibited by treatment with N-acetylcysteine (NAC), a powerful antioxidant (Figure 5C). These results suggested that CD44 upregulation in gastric cancer cells may be enhanced through miR-328 downregulation by oxidative stress in the tumor microenvironment. Macrophages are known to produce ROS during phagocytosis and in response to various stimuli, and macrophage-generated oxidants may act as carcinogens causing both tumor initiation and promotion (25). Thus, we investigated if macrophage-derived ROS affected the expression of miR-328 and CD44
in gastrointestinal cancer cells. We co-cultured M1- and M2-polarized THP-1 macrophages with low-CD44-expressing AGS gastric cancer cells (Supplementary Figure S3A, available at Carcinogenesis Online) (26). Consistent with previous research, M1- and M2-polarized THP-1 macrophages showed a distinct cytokine-production profile (e.g. IL-12, IL-1β, TNFα, IL-6; Supplementary Figure S3B, available at Carcinogenesis Online). M1- and M2-polarized THP-1 macrophages were then co-cultured with AGS cells for 24 h and examined by fluorescence microscopy after staining with the ROS-sensitive fluorescent probe 2',7'-dichlorofluorescein diacetate. M1- and M2-polarized macrophages showed abundant ROS and pronounced 2',7'-dichlorofluorescein diacetate staining (Supplementary Figure S3C, available at Carcinogenesis Online). Infiltration of CD68+ macrophages correlated with high CD44v9 expression and low miR-328 expression in gastric cancer cells (Figure 6A and B), whereas infiltration of CD163+ macrophages correlated with high CD44v9 expression and low miR-328 expression (Figure 6C and D). These results suggest that the number of infiltrated macrophage in the tumor stroma was significantly associated with CD44 upregulation and miR-328 downregulation in patients with gastric cancer.

**Fig. 4. miR-328 decreases resistance to ROS and chemotherapeutic drug and suppresses cancer cell growth in vivo.** (A) HCT116 cells transfected with control or mimic miR-328 were incubated for 72 h with the indicated concentrations of H2O2 or CDDP and then assayed for cell viability. Data are expressed as treated/control cell ratio and are mean ± SD from triplicate experiments. The median inhibitory concentration (IC50) values are also shown. (B) HCT116 cells co-transfected with mimic miR-328 + mock vector or mimic miR-328 + CD44v8-10 were incubated for 72 h with the indicated concentrations of H2O2 or CDDP and assayed for cell viability. Data are expressed as treated/control cell ratio and are given as mean ± SD from triplicate experiments. The median inhibitory concentration (IC50) values are also shown. (C) Immunoblot analysis of CD44 in HCT116 cells stably transfected with mock or miR-328 vector. (D) Weights of tumors formed by HCT116 cells stably transfected with control or miR-328 vector were determined at 35 days after cell injection. Data are given as mean ± SD for five animals in each group. *P < 0.01.
Discussion

We previously reported that CD44 is not only a major CSC marker but also plays a functional role in ROS defense, resulting in tumor development and colonization of metastatic cancer cells (8,27). Persistent ROS stress may induce adaptive stress responses, including the upregulation of redox-sensitive transcription factors, antioxidants and survival factors. Redox adaptation not only enables cancer cells to survive under increased ROS stress but also renders them resistant to certain anticancer drugs (28,29). CD44-related signaling thus provides a potential therapeutic target, but the mechanisms regulating CD44 expression in cancer cells remain incompletely understood. Several miRNAs suppress CD44 expression by targeting the CD44 3′-UTR, resulting in suppression of tumorigenesis and metastasis. For instance, miR-34a is a key negative regulator of CD44+ prostate CSCs and inhibits metastasis by directly repressing CD44, whereas miR-199a targets CD44 to suppress tumorigenicity and multidrug resistance in CD44+CD117+ ovarian cancer-initiating cells (30,31). Based on these previous studies, we speculated that miRNAs may be a key factor in CD44 upregulation in gastrointestinal cancer cells. We, therefore, performed miRNA screening and identified the candidate miRNA, miR-328, in gastrointestinal cancer cell lines. The results of this study demonstrated that miR-328 modulated CD44 expression by directly targeting the CD44 3′-UTR and identified the miR-328-targeting sequences within the CD44 3′-UTR (32). We examined three candidate miR-328 binding sites (353–374, 844–865 and 943–965) in the CD44 3′-UTR using the miRanda algorithm and identified the miR-328 target sequence (353–374). This study provides the first characterization of this target sequence. Furthermore, we clarified that miR-328 expression affected cancer cell growth and chemotherapeutic drug and ROS resistance. CD44 expression in gastric cancer cells was triggered by ROS and inflammatory cytokines, and oxidative stress by H2O2 suppressed miR-328 expression in a concentration-dependent manner. However, TNFα had no effect on miR-328 expression in gastric cancer cells. Thus, although this study provides evidence that miR-328 expression is modulated by oxidative stress, the precise mechanism of miR-328 regulation remains unknown. Previous studies have shown that miRNA expression can be deregulated by epigenetic alterations, including aberrant DNA methylation, and such mechanisms may be implicated in the regulation of miR-328 by oxidative stress (33,34).
Therefore, further investigations are needed to reveal the mechanism of miR-328 suppression by oxidative stress.

In cancer tissues, ROS is produced by a variety of cell types, including neutrophils, macrophages and endothelial cells. We recently showed that ablation of CD44 in the Gan mouse genetic gastric cancer model, triggered tumor-cell growth arrest through ROS-mediated signals, resulting in tumor suppression. Abundant macrophages infiltrated into the tumor stroma in Gan mouse tumors, and these macrophages have been reported to contribute to CD44 expression and gastric tumor progression through inflammatory cytokines (35,36). In this study, we revealed that infiltrated macrophages in the tumor microenvironment may contribute to redox adaptation through CD44 upregulation and miR-328 downregulation. Previous studies have shown that M2 macrophages are associated with a poorer prognosis in various types of cancer (18,37,38); however, M2 macrophages are correlated with a good prognosis in gastrointestinal cancer (39,40), suggesting that the different functional roles of M1- and M2-polarized macrophages depend on the state of the cancer cells and the organ. Furthermore, recent studies have provided evidence indicating functional plasticity of M1/M2 macrophages in response to different microenvironmental signals (41,42). The present results indicated that both of M1- and M2-polarized macrophages triggered CD44 expression through miR-328 suppression, suggesting no difference between these two types of macrophages in terms of miR-328 suppression and CD44 induction. The number of infiltrated macrophages was also significantly associated with CD44 upregulation and miR-328 downregulation in gastric cancer patients. These findings reveal that infiltrated macrophages in the tumor microenvironment cause CD44 upregulation through miR-328 suppression. CD44 is implicated in redox adaptation, resulting in cancer cell growth and resistance to chemotherapeutic drugs through enhanced ROS defense.

In conclusion, the results of this study reveal a new redox adaptation mechanism in gastrointestinal cancer and indicate that miR-328-CD44 signaling mediated by macrophages may represent a novel therapeutic target for gastrointestinal cancer treatment.

Supplementary material
Supplementary Tables S1, S2 and Figures S1–S3 can be found at http://carcin.oxfordjournals.org/

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References

Fig. 6. Correlations between the numbers of CD68+ and CD163+ macrophages and upregulation of CD44 expression and downregulation of miR-328 in human gastric cancer. (A) Left images show CD44v9 and CD68 expression patterns in gastric cancer. Scale bars, 100 μm. Right panel shows the correlation between CD44v9 expression status in gastric cancer cells and CD68-expressing macrophages in gastric cancer stroma. There was a significant correlation between these two groups (*P < 0.01). (B) Expression of miR-328 in gastric stroma tissues with high and low levels of CD68 macrophages. There was a significant difference in miR-328 expression between these two groups (*P < 0.01). (C) Left images show CD44v9 and CD163 expression patterns in gastric cancer. Scale bars, 100 μm. Right panel shows correlation between CD44v9 expression status in gastric cancer cells and CD163-expressing macrophages in gastric cancer stroma. There was a significant correlation between these two groups (*P < 0.03). (D) Expression of miR-328 in gastric stroma tissues with high and low levels of CD163 macrophages. There was a significant difference in miR-328 expression between these two groups (*P < 0.03).
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